A Molecular Defect in Two Families with Hemolytic Poikilocytic Anemia

REDUCTION OF HIGH AFFINITY MEMBRANE BINDING SITES FOR ANKYRIN

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ABSTRACT Patients from two families with chronic hemolytic anemia have been studied. The erythrocytes are very fragile and appear microcytic with a great variety of shapes. Clinical evaluation failed to identify traditionally recognized causes of hemolysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed no significant abnormality of the major polypeptide bands. Erythrocytes spectrin-ankyrin and ankyrin-membrane interactions were analyzed with ^{125}I-labeled spectrin, ^{125}I-labeled ankyrin, and inside-out vesicles. Patients' vesicles bound ^{125}I-spectrin normally. Likewise, patients' spectrin and ankyrin competed normally for the binding sites on control membranes. None of the individual components appeared to have abnormal thermal sensitivity. Ankyrin-stripped, inside-out vesicles prepared from the patients bound less ^{125}I-ankyrin than did vesicles prepared from normals ($P < 0.05$ for all corresponding points in the high-affinity region). Scatchard analysis showed the most significant abnormality to be a 50% reduction in the high affinity ankyrin binding sites. Similar experiments were performed with blood from patients with spherocytosis and splenectomized controls, but no abnormalities were detected. The water soluble 43,000-dalton fragments of band 3 (the high-affinity ankyrin binding sites) were prepared from one of the patients and competed normally for ^{125}I-ankyrin binding in solution. This suggests that the primary structural defect is a reduction in the number of high affinity membrane binding sites for ankyrin, and is consistent with an abnormal organization of band 3 in the membrane.

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INTRODUCTION

The molecular structure of the erythrocyte membrane has been extensively studied (1–3). It contains a membrane-associated cytoskeleton responsible for the unusual stability of this cell in circulation, and it retains the shape of the original cell after extraction with nonionic detergents (1, 4). The cytoskeleton is composed of spectrin, band 4.1[1] and erythrocyte actin, as well as some other associated polypeptides. Ghosts rapidly disintegrate into small vesicles after selective extraction of spectrin and actin (1). Spectrin has been shown to exist on the membrane as a tetramer consisting of two dimers associated head to head (5, 6), and ghosts with spectrin principally in the dimer form are unstable (7). Spectrin has also been shown to bind to erythrocyte actin and band 4.1 (8–10). Spectrin dimers have been visualized by low angle rotary shadowed

[1] Nomenclature of Steck (1).

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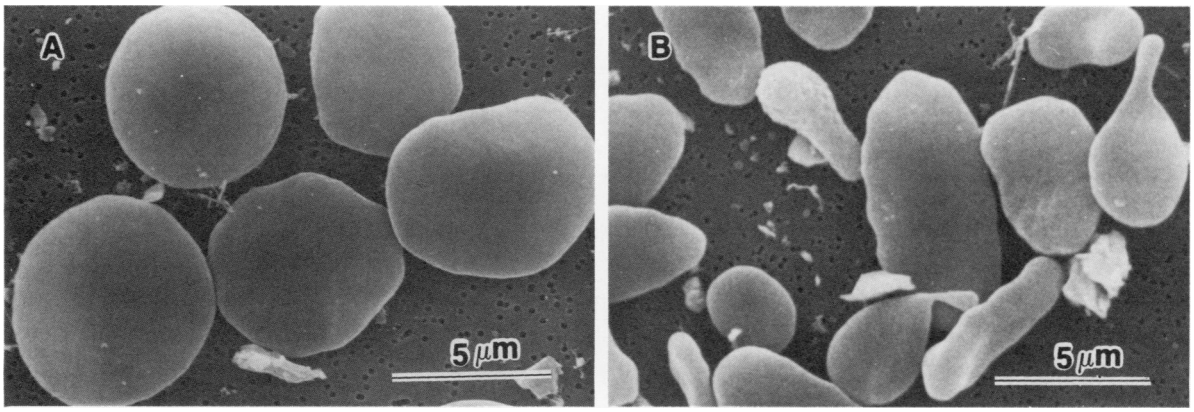

FIGURE 1 Scanning electron micrographs of erythrocytes from a normal individual (A) and hemolytic patient A.P. (B).

electron microscopy with identification of binding sites for ankyrin, band 4.1, and actin as well as the site of tetramer formation (6, 11). Functional domains of spectrin have been identified by peptide fragment analysis (12).

Abnormalities of spectrin are sometimes associated with gross abnormalities in erythrocyte shape. Mutant strains of mice have been reported that are deficient in spectrin and have extremely fragile spherocytic erythrocytes (13, 14). Pyropoikilocytosis is a disorder of erythrocytes characterized by abnormal temperature-sensitive cell fragmentation (15), and the spectrin has been shown to have increased sensitivity to thermal denaturation (16). Some kindreds with elliptocytosis also appear to have spectrin with slightly increased heat sensitivity (17), and spectrin from a similar patient was shown to yield abnormal trypsin digestion fragments (18). Other reports indicate that other forms of elliptocytosis may be due to alterations in spectrin tetramer-dimer equilibrium (19) or reductions in the amount of band 4.1 (20, 21).

Direct association of cytoskeletal proteins with membrane components has been demonstrated (22–24). Spectrin binds with high affinity to band 2.1, a polypeptide localized on the inner surface of the membrane named "ankyrin" (25–28) which has been purified (11, 29). Ankyrin binds directly to the membrane at a site identified as the cytoplasmic domain of band 3 (30). We searched for defects in this linkage in abnormally shaped erythrocytes. Two families were located with hemolytic poikilocytic anemia, and reductions in the number of high affinity ankyrin binding sites were identified in each.

METHODS

Patients

Individuals from two unrelated families were studied in detail. Affected members had varying degrees of icterus and anemia and their erythrocytes have bizarre morphology (Fig. 1). Clinical evaluation ruled out abnormalities of hemoglobin, glycolytic enzymes, autoimmunity, and nutrition because the following determinations were normal: serum iron, transferrin, ferritin, vitamin B_{12} and folate, free erythrocyte protoporphyrin, hemoglobin electrophoresis, α:β-globin ratios, glycolytic enzymes, direct and indirect Coombs, and fibrin degradation products. Preliminary heat-induced fragmentation studies showed no significant temperature shift. Studies with ^{51}Cr-labeled erythrocytes from patient D.W. before splenectomy showed a survival of 15 d (normal 26–35 d). Cells from patients had slightly increased hypotonic lysis before splenectomy (midpoint 0.48% NaCl, normal 0.39–0.46%) and greatly increased hypotonic lysis after splenectomy (midpoint 0.57% NaCl). Increased autohemolysis was noted (7.8 and 10.8%, normal <3%) and was partially corrected with glucose (1.9 and 2.7%, normal <1%). All three patients underwent splenectomy, which resulted in an improvement of the anemia (Table I), although the abnormal erythrocyte morphology persisted.

North Carolina family. The index case, A.P., is a 23-yr-old black male with chronic anemia who first required a blood transfusion at age 5 yr. Scleral icterus was noted intermittently, and he was referred to the University of North Carolina at age 18. He had splenomegaly but looked well. He underwent splenectomy and now enjoys excellent health. A younger sister (H.P.) has similar erythrocytes and recently underwent splenectomy with cholecystectomy resulting in clinical improvement. An older brother (H.W.P.) has similar erythrocytes but compensated hemolysis. The other three siblings and both parents are healthy and have normal erythrocytes. Mistaken paternity has been excluded with a survey of genetic markers. Also, inheritance of Rh antigens was not clearly linked with the erythrocyte defect, and sulfate flux studies were normal.

Ontario family. The index case, D.W., is a 65-yr-old Caucasian woman with chronic hemolytic poikilocytic anemia that was exacerbated by gastrointestinal bleeding 14 yr ago. After splenectomy she improved clinically and enjoys good health. Her son (P.W.) was found to have uniformly elliptocytic erythrocytes, but is not significantly anemic. None of her other relatives have histories of blood disorders, and all of those checked have normal peripheral blood smears.

Control blood samples were also obtained from many unrelated individuals including several healthy adults with normal erythrocytes, five adults with varying degrees of

TABLE I
Summary of Clinical Data

Family and race	Patient	Hemoglobin: Pre-splenectomy	Hemoglobin: Post-splenectomy*	Reticulocytes: Pre-splenectomy	Reticulocytes: Post-splenectomy*	MCV‡	Erythrocyte morphology
		g/100 ml		%			
North Carolina Black	A.P.§	10.4	13.4	13.6	2.2	56	Marked poikilocytosis, anisocytosis, and microcytosis
	H.P. (sister)	9.0	13.3	7.6	1.9	58	Similar to A.P.
	H.W.P.‖ (brother)	13.3		7.2		73	Similar to A.P.
	Mother	12.5		1.6		81	Normal
	Father	12.5		1.3		84	Normal
Ontario Caucasian	D.W.§	7.5	10.5	11.4	2.3	63	Marked poikilocytosis, anisocytosis, and microcytosis
	P.W. (son)	13.1		2.3		85	Elliptocytosis
	B.W. (son)	14.0				86	Normal
	J. B. (daughter)	13.1				88	Normal

* Blood drawn at least 6 wk after splenectomy.
‡ Mean corpuscular volume, fluid.
§ Index case of family.
‖ H.W.P. subsequently developed hepatitis and was not included in the ankyrin binding studies.

spherocytosis, and three adults who had undergone splenectomies because of other problems.

Materials

^{125}I-Bolton-Hunter Reagent (2,000 Ci/mmol) was from New England Nuclear, Boston, Mass. α-Chymotrypsin (70 U/mg) was from Worthington Biochemicals, Freehold, N. J. Acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), and Coomassie Brillant Blue were from Bio-Rad Laboratories, Richmond, Calif. DEAE-cellulose (DE 52) was from Whatman Inc., Clifton, N. J.

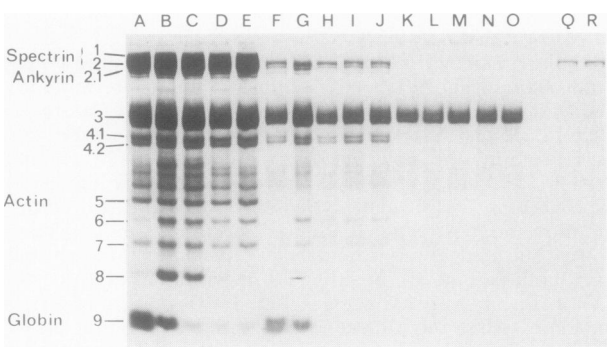

FIGURE 2 Membrane preparations from the North Carolina family were analyzed by SDS-PAGE on 7% 3-mm thick slab gels and stained with Coomassie Blue. Approximately 50 μg protein of erythrocyte ghosts were applied (lanes A–E), ~25 μg protein of inside-out vesicles (lanes F, H–J, but note ~35 μg protein in lane G), 15 μg protein of ankyrin-stripped inside-out vesicles (lanes K–O), and 3 μg protein of partially purified ankyrin (lanes Q and R). The corresponding preparations from patient A.P. are in lanes B, G, L, and R, patient H.P. in lanes C, H, and M, their mother (normal RBC) in lanes A, F, and K, their father (normal RBC) in lanes D, I, and N, and an unrelated normal individual in lanes E, J, O, and Q.

Preparative methods

Venous blood samples were anticoagulated with acid citrate-dextrose and stored at 0°C for up to 24 h. Washed erythrocytes were hypotonically lysed as described (27). The viscous leukocyte pellet was carefully removed by aspiration, and the ghosts were washed repeatedly until those from normal cells were white. Ghosts from patients with each of the various hemolytic anemias usually remained slightly pink unless washed more extensively. Inside-out vesicles depleted of spectrin and actin and ankyrin-stripped, inside-out vesicles were prepared taking precautions to avoid proteolysis as described (25, 26, 31, 32).

Spectrin heterodimer was purified to homogeneity from erythrocytes of normal individuals as described (31, 33). Pure spectrin was radiolabeled with ^{125}I-Bolton-Hunter reagent essentially as described for radiolabeling ankyrin (32). After dilution with pure unlabeled spectrin, the specific activities of the different preparations ranged between 70,000 and 285,000 cpm/μg. Ankyrin was purified to homogeneity from the erythrocytes of normal individuals as described (29) and was radiolabeled with ^{125}I-Bolton-Hunter reagent (32). After dilution with unlabeled ankyrin, the final specific activities ranged between 50,000 and 90,000 cpm/μg. Note that in all experiments ^{125}I-spectrin and ^{125}I-ankyrin refer to radiolabeled pure protein prepared from normal individuals. Ankyrin for competition experiments was partially purified by selective extraction of the cytoskeletons.

Assay methods

Binding of ^{125}I-spectrin to inside-out vesicles was measured as reported (31). Samples of ^{125}I-spectrin were heat-denatured (70°C, 10 min) and were tested at every ^{125}I-spectrin concentration for each patient in each experiment to estimate nonspecific binding. Such measurements showed ~10% of the total counts per minute bound to be nonspecific, and the measured value was routinely subtracted. All spectrin binding experiments are presented with each point representing duplicates and had ranges of $<\pm 6\%$.

Binding of ^{125}I-ankyrin to ankyrin-stripped, inside-out vesicles was measured as reported (32). ^{125}I-Ankyrin (1–50 μg, 50,000 to 90,000 cpm/μg) and ankyrin-stripped, inside-out vesicles (6–13 μg membrane protein) were incubated in 0.2 ml vol for 150 min at 24°C. Free and membrane-bound [^{125}I]-ankyrin were separated and nonspecific binding (which was generally ~10% of the total) was measured for each point and corrected as described above. All values represent duplicates or triplicates and had ranges of $<\pm 6\%$. The ^{125}I-ankyrin binding data were fitted to the four parameter Scatchard model (34, 35) with Scatchard model test data, a nonlinear computer analysis (36).

Scanning electron microscope studies were performed on aliquots of the blood samples that were diluted into prefiltered 0.1 M sodium phosphate, 3% glutaraldehyde, pH 7.4 immediately after blood-drawing. Membrane protein was estimated (37) using bovine serum albumin as the standard. Spectrin, ankyrin, and 43,000-dalton fragments of band 3 were estimated by absorbance at 280 nM. SDS-polyacrylamide gel electrophoresis (PAGE) was performed essentially by the method of Fairbanks (38).

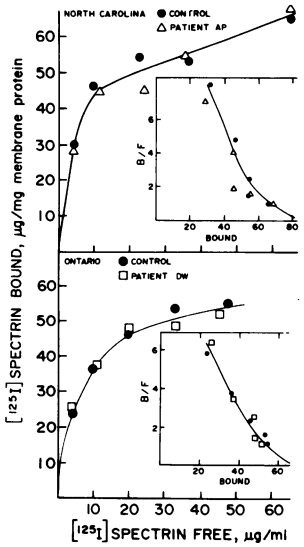

FIGURE 3 Effect of increasing the concentration of ^{125}I-spectrin on binding to inside-out vesicles. The ^{125}I-spectrin, in this and all experiments, was prepared from a normal individual. The inside-out vesicles were prepared from patients (△, A.P., N. C., above; and □, D.W., Ontario, below) and normals (●). Various concentrations of ^{125}I-spectrin were incubated at 0°C in a 0.2-ml vol with the corresponding inside-out vesicles (9 μg membrane protein). Specifically membrane bound and free radioactivity were determined for each point. These data are plotted according to the Scatchard equation in the insets.

RESULTS

SDS-PAGE was performed on membranes and vesicles at each stage of preparation, and there is no significant abnormality of the major polypeptide bands (Fig. 2). In the ghosts (lanes A–E) band 8 and hemoglobin appear increased in patient preparations but can be reduced by more extensive washing. This is also seen in ghosts from several other sorts of hemolytic anemias and probably represents adherent protein rather than protein trapped in right-side-out vesicles. Spectrin-stripped, inside-out vesicles (lanes F–J) appear equivalent. Ankyrin-stripped, inside-out vesicles (lanes K–O) consisted of identical amounts of band 3 and periodic acid-Schiff (PAS) staining (not shown), which are the principal polypeptides remaining. Special care was taken to detect residual ankyrin on the vesicles, but the tiny amount (<5%) of spectrin and ankyrin remaining did not correlate with reductions of [^{125}I]-ankyrin binding. Spectrin (not shown) and ankyrin were partially purified from each of the patients and controls, and no differences in quantity or electrophoretic mobility were noted. Proteolysis was a concern so precautions were taken to avoid it, and inspection of the gels failed to demonstrate evidence of any proteolysis. SDS-PAGE of preparations from members of the Ontario family were also essentially normal (not shown).

Spectrin binding sites are normal. The binding sites for spectrin on the membrane (ankyrin) were examined by comparing the capacities of patient and control inside-out vesicles to bind ^{125}I-spectrin heterodimer prepared from normal erythrocytes (Fig. 3). Increasing concentrations of ^{125}I-spectrin were incubated with constant amounts of inside-out vesicles, and the total binding capacities and affinities were identical. Although higher capacities were measured in other experiments, the values obtained from patients and controls were always identical within each experiment, which indicates that there is no significant difference in the spectrin binding sites.

Binding of spectrin and ankyrin is normal. Various concentrations of unlabeled spectrin (from patients or controls) were incubated with a constant amount of ^{125}I-spectrin and a constant amount of control inside-out vesicles. Unlabeled spectrin competed with ^{125}I-spectrin for the binding sites, and spectrin samples from patients and controls appeared equivalent (Table II). Similar experiments were also performed with unlabeled ankyrin, and patient and control preparations again appeared equivalent (Table II). Spectrin from patients with hereditary pyropoikilocytosis (16) is more sensitive to heat denaturation than normal spectrin. Spectrin from patient A.P. and a control were preincubated at several temperatures above 50°C with identical loss of binding ability. Likewise, ankyrin from

TABLE II
*Competitive Inhibition of ^{125}I-Spectrin and ^{125}I-Ankyrin Binding**

Unlabeled protein	μg/ml	Family	Patient	Inhibition of binding
				%
Spectrin	41.0	N. C.	A.P.	46.6±4.3
			P.A.	44.5±0.7
	52.0	Ont.	D.W.	55.1±4.0
			D.C.	50.7±0.2
Ankyrin	54.5	N. C.	A.P.	54.2±4.4
			P.A.	52.7±4.5
	48.1	Ont.	D.W.	48.6±2.4
			D.C.	48.8±2.2

* Binding of ^{125}I-spectrin (1 μg) to inside-out vesicles (20 μg protein) was measured (0.2 ml volume, 90 min, 0°C), in the presence and absence of unlabeled spectrin prepared from patients A.P. and D.W. and unrelated normals P.A. and D.C. Similarly, binding of ^{125}I-ankyrin (0.4 μg) to ankyrin-stripped, inside-out vesicles (10 μg protein) was measured (0.2 ml volume, 150 min, 24°C) in the presence and absence of unlabeled ankyrin. The percent inhibition is derived from the amount of ^{125}I-spectrin (or ^{125}I-ankyrin) specifically bound in the presence of unlabeled protein divided by that bound in the absence. The error is ±0.5 the range of duplicates. Measurements were also made at several other concentrations of unlabeled spectrin and anykyrin, and patient and control preparations appeared identical (data not shown).

patient A.P. and a control had identical loss of binding ability when preincubated at several temperatures above 42.5° as did their ankyrin-stripped, inside-out vesicles (data not shown). Thus spectrin and ankyrin from patient cells reassociate in a normal manner with their respective binding sites and exhibit no change in thermal sensitivity.

Ankyrin binding sites are abnormal. Experiments were conducted to compare the ability of ankyrin-stripped, inside-out vesicles (prepared from different patients) to bind ^{125}I-ankyrin (prepared from a normal). The vesicles prepared from patients of both families bound 24–49% less ^{125}I-ankyrin than vesicles prepared from unaffected family members or unrelated controls (Table III). This was reproducible in multiple determinations performed on blood samples obtained from patients and many controls on several occasions. Both of the North Carolina patients had reductions in the ^{125}I-ankyrin binding, but both parents had normal ^{125}I-ankyrin binding. The Ontario patient, D.W., had consistently reduced ^{125}I-ankyrin binding. Of note, P.W., her son with classical elliptocytosis had nearly normal ^{125}I-ankyrin binding as did the other siblings that were analyzed. ^{125}I-Ankyrin binding was normal in vesicles from two patients with spherocytosis and three splenectomy patients. It appears that microcytosis, high reticulocyte counts, and absence of the spleen are not the factors responsible for reduced ankyrin binding.

Detailed binding experiments were conducted over wide ranges of ^{125}I-ankyrin concentrations. Plots of binding data (Fig. 4, left panels) show significantly reduced ^{125}I-ankyrin binding for all points in the high affinity region of each profile. Data from these individuals were analyzed with a computer program for unconstrained nonlinear regression analysis of unweighted data in order to resolve curvilinear Scatchard plots (Fig. 4, middle panels) into linear components of the best fit (Fig. 4, right panels) with estimation of the errors (36). This permits evaluation of differences between patient and control data and identification of the parameter(s) (B_1, K_{D1}, B_2, or K_{D2}) most responsible for the notable reduction in ^{125}I-ankyrin binding. Each binding profile was resolved into two lines: one with high affinity (steeper slope → smaller K_{D1}) but low capacity (smaller intercept on the abscissa = B_1), and one with low affinity (less steep slope → larger K_{D2}) but high capacity, (larger intercept on the abscissa = B_2).

Data from these and other experiments are summarized in Table IV. Estimates of B_1 for all normal individuals from the different experiments varied from 21.0 to 31.0 μg/mg (mean±SD = 25.9±3.5). Estimates of B_1 for patients A.P., H.P., and D.W. were always lower than for normals and ranged from 8.4 to 13.7 in the different experiments (mean±SD = 10.6±2.2). Note that patient A.P. and patient H.P. had similar values even though A.P. had undergone a splenectomy and, at this time, H.P. had not. The statistical significance of each of these differences in B_1 were determined within experiments 8 and 10: $P < 0.10$ for A.P., $P < 0.12$ for H.P., and $P < 0.006$ for D.W., when calculated from a normal distribution (Table IV).

Values of the other parameters (K_{D1}, B_2 and K_{D2}) were not consistently different when calculated for patients and controls. Points in the low affinity region of the Scatchard plots (points with >40 μg/mg bound) approach the abscissa asymptotically and are vulnerable to significant error. When the ^{125}I-ankyrin concentrations selected did not result in points of >40 μg/mg bound, one cannot accurately determine B_2 and K_{D2} (see experiments 9A and 9B in Table IV). Nevertheless, while experiment 8 indicates that there could be some reduction in capacity of the low affinity sites (B_2), the estimates contain very wide standard deviations, and therefore reduction of B_2 is of little statistical significance. Indeed, not only is there variation of B_2 within an experiment, there are large differences between experiments (8, 10, Table IV). Because B_2 is invariably much larger than B_1, the total ankyrin binding capacity ($B_1 + B_2$) cannot be considered signifi-

TABLE III

*^{125}I-Ankyrin Binding Determinations**

Exp.	Family	Patient‡		[^{125}I]Ankyrin bound§
				% Control
1	N. C.	A.P.		51±0
		P.A.	Unrelated, normal	100±3
2	N. C.	A.P.		76±0
		H.P.		70±2
		Mother		106±1
		Father		106±4
		P.A.	Unrelated, normal	100±5
3	N. C.	A.P.		68±1
		C.H.	Unrelated, splenectomy	94±6
		P.A.	Unrelated, normal	103±1
		M.C.	Unrelated, normal	99±0
		J.D.	Unrelated, normal	100±4
		G.T.	Unrelated, normal	104±3
4	Ont.	D.W.		52±4
		M.S.	Unrelated, splenectomy	94±6
		D.C.	Unrelated, normal	100±1
5	Ont.	D.W.		71±2
		P.W.	Son, elliptocytosis	100±1
		J.B.	Daughter, normal	105±3
		B.W.	Son, normal	102±3
		D.C.	Unrelated, normal	100±1
6	Controls	H.S.	Unrelated, spherocytosis	91±6
		S.W.	Unrelated, spherocytosis	105±4
		P.A.	Unrelated, normal	100±6
7	Controls	L.R.	Unrelated, splenectomy	98±1
		J.D.	Unrelated, normal	100±1

* These determinations are derived from duplicate values from seven preliminary experiments. Other similar binding experiments with similar results were obtained from vesicles prepared from blood samples drawn from members of both families as well as many unrelated individuals.

‡ Patients with hemolytic poikilocytosis are listed by initials only (A.P., H.P., and D.W.). Other family members are identified. Unrelated normals are all adults with no medical problems. Other unrelated controls are identified and include three persons with normal erythrocytes who had splenectomies for other reasons. (M.S., C.H., and L.R.) and two persons with moderately severe hereditary spherocytosis (H.S. and S.W.).

§ Ankyrin binding was performed as described for Fig. 4. In each experiment, all individual determinations were done with identical membrane protein concentrations and identical ^{125}I-ankyrin concentrations. These are normalized to the value obtained from the unrelated normal individual in each experiment (or mean of the values obtained from four normal individuals in experiment 3) ±0.5 of the range of the duplicates.

cantly different when the different patients and controls are compared, and the total capacity may even be the same. Determination of K_{D1} and K_{D2} invariably contained wide standard deviations so differences were of no statistical significance.

Statistical analysis of curvilinear Scatchard plots is fraught with difficulty. Another method has also been used to assess the contributions of capacity and affinity to apparent differences between two plots (39). When our data were analyzed with this method also, the reduction in B_1 of our patient data greatly outweighed changes in K_{D1} in explaining the differences between patient and control data.

In summary, ankyrin-stripped, inside-out vesicles prepared from patients A.P., H.P., and D.W. always had significant reductions in the ability to bind ^{125}I-ankyrin. Computer analysis showed the most statistically significant explanation to be a 50% reduction in

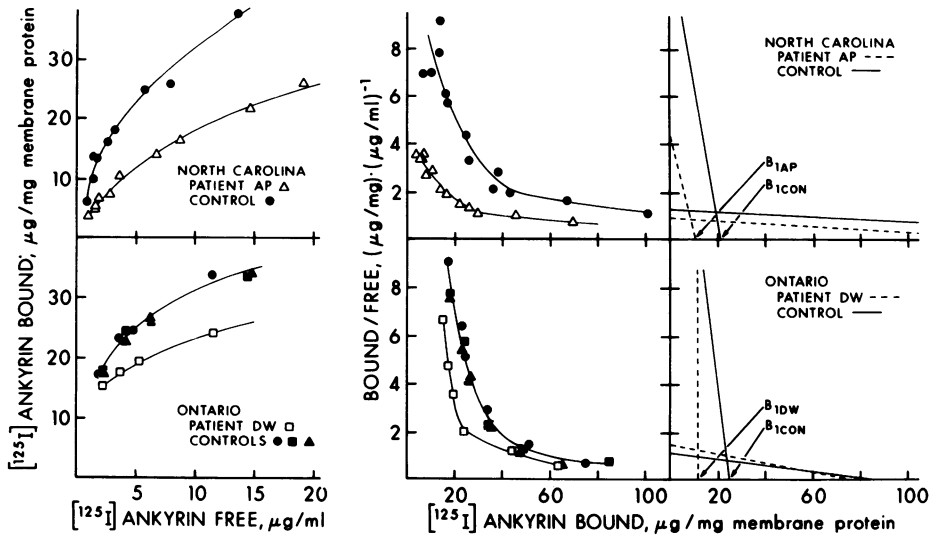

FIGURE 4 Effect of increasing the concentration of ^{125}I-ankyrin on binding to ankyrin-stripped inside-out vesicles. ^{125}I-Ankyrin, in this and all experiments, were prepared from normal individuals. The inside-out vesicles were prepared from patients (△, A.P., and □, D.W.), unrelated normal individuals (●), and normal family members (▲, ■). Various concentrations of [^{125}I]ankyrin were incubated for 150 min at 24°C in 0.2 ml vol with 10 μg protein of the corresponding ankyrin-stripped inside-out vesicles. Specifically membrane-bound and free radioactivity were determined for each point. The data for the points in the high affinity region (<40 μg/mg bound) are plotted in the panels on the left as bound vs. free. The patient values are significantly different from normals (ranging from $P < 0.01$ to $P < 0.05$). The data for all points are plotted according to the Scatchard equation in the panels in the middle. Each curve appears to be biphasic with, a low capacity high affinity region and a high capacity low affinity region. Values for these experiments and others were analyzed by computer to resolve the curvilinear plots into the individual parameters. These are plotted in the panels on the right as solid lines for controls and broken lines for patients and are summarized in Table IV. The number of high affinity sites are indicated on the abscissa with arrows for patients (B_{1AP} and B_{1DW}) and for normals (B_{1CON}).

the high affinity binding sites, B_1, but without consistent changes in the actual affinity of the interaction (K_{D1}) or in the affinity (K_{D2}) or capacity (B_2) of the low affinity binding sites. The lack of statistically significant differences in the total ankyrin binding capacity ($B_1 + B_2$) is consistent with the similarities of the total content of ankyrin and band 3 of patient membranes as well as similarities in spectrin binding.

Ankyrin binding sites are normal in solution. Ankyrin has been shown to bind to the erythrocyte membrane with high affinity through a specific interaction with the cytoplasmic portion of the band 3 molecule (29, 32, 40). Controlled enzymatic digestion of ankyrin-stripped, inside-out vesicles with α-chymotrypsin cleaves a water soluble 43,000-dalton fragment from the cytoplasmic portion of band 3. This polypeptide has been purified and has been shown to bind to ankyrin in solution. Indeed, the soluble peptide will compete with membrane-associated band 3 for binding of ^{125}I-ankyrin in solution. When the 43,000-dalton ankyrin binding fragment of band 3 was prepared from patient A.P. and a normal control, the preparations contained identical amounts of protein. Furthermore, both fragment preparations inhibited the binding of ^{125}I-ankyrin to membranes equivalently. Thus, the abnormality on the membrane is no longer demonstrated when the binding sites are in solution.

DISCUSSION

Similar specific defects were found in the membranes of poikilocytic erythrocytes of three patients from two different families. The erythrocyte morphology of each is strikingly abnormal, yet the patients are all in good health. It was anticipated that the actual molecular defect would be subtle since an absolute dysfunction of an essential membrane structure would probably lead to much more severe hemolysis. Spectrin and spectrin binding sites (ankyrin) appeared normal. There was no peculiar heat sensitivity of spectrin, ankyrin, or membrane vesicles. However, ankyrin-stripped, inside-out vesicles prepared from these patients bound significantly less than normal amounts of ^{125}I-ankyrin. Scatchard analysis indicated that the most likely explanation is a 50% reduction in the number of high affinity ankyrin binding sites on the

TABLE IV

*Scatchard Analysis of ^{125}I-Ankyrin Binding Experiments**

				High affinity sites					Low affinity sites			
				B_1			K_{D1}		B_2		K_{D2}	
Exp.‡	Family	Patient	n§	Estimate‖	%	P¶	Estimate**	P	Estimate‖	P	Estimate**	P
8	N. C.	A.P.	30	10.8±4.2	(51)	<0.1	2.55±1.60	NS	169±45	NS	179±87	NS
		H.P.‡‡	30	8.8±6.4	(42)	<0.12	0.85±1.61	NS	142±21	<0.16	75±31	NS
		P.A.§§	30	21.0±4.4	(100)		1.73±0.68		271±82		212±108	
9A	N. C.	A.P.	12	8.4±19.7	(30)	‖‖	0.01±2.56	‖‖				
		J.D.§§	12	29.7±10.7	(100)		1.49±0.97					
9B	N. C.	A.P.	16	13.7±12.0	(54)	‖‖	0.57±1.65	‖‖				
		G.T.§§	16	23.3±13.6	(92)	‖‖	1.29±1.29	‖‖				
		P.A.§§	16	25.4±26.8	(100)		1.60±2.48					
10	Ont.	D.W.	12	11.5±3.1	(46)	<0.006	0.01±0.62	<0.13	77±4	NS	51±12	NS
		P.W.	12	21.9±7.2	(88)	NS	0.94±1.29	NS	169±92	NS	209±204	NS
		B.W.	12	25.8±3.4	(104)	NS	1.27±0.50	NS	75±7	NS	89±30	NS
		J.B.	12	31.0±5.5	(125)	NS	2.06±0.84	NS	90±34	NS	162±143	NS
		D.C.§§	12	24.8±3.5	(100)		1.19±0.46		86±6		74±19	

* Experiments are described in the legend of Fig. 4. Data were analyzed by Scatchard model test data, nonlinear computer analysis (36). This allowed calculation of estimates (B_1, B_2, K_{D1}, K_{D2}) and standard deviations.

‡ Although different batches of control ^{125}I-ankyrin were used in different experiments, they were prepared identically. Specific activities were 53,600 cpm/μg in experiment 8; 72,500 in 9A and 9B; and 87,800 in 10. Binding was measured over wide ranges of ^{125}I-ankyrin concentrations, but with constant amounts of ankyrin-stripped, inside-out vesicles. Data from experiments 8 and 10 are plotted in Fig. 4 top and bottom.

§ *n*, number of individual points measured per binding curve.

‖ B_1 and B_2, micrograms of ankyrin bound per milligram of membrane protein±SD. The percentage (in parentheses) refers to the patient value when compared with the normal value.

¶ *P* values of difference calculated from a normal distribution, NS ≥ 0.20.

** K_{D1} and K_{D2}, micrograms of ankyrin bound per milliliter±SD; multiply by 4.7 to calculate in terms of nanomolarity.

‡‡ H.P. underwent a splenectomy subsequent to these determinations.

§§ Unrelated normal individual.

‖‖ In experiment 9A and 9B, all points contained <305 μg/ml of ^{125}I-ankyrin. Most of these points are in the high affinity area of the Scatchard plot (B < 40 μg/mg). Therefore, the SD for B_1 and K_{D1} are too wide for reasonable calculation of probability. Also, the lack of points in the lower affinity portion of the binding plots make the calculated B_2, and K_{D2} very unreliable. Nevertheless, estimates of B_1 agree with the values measured in experiment 8.

membrane. However, when the binding sites were cleaved from the membrane, identical associations of ankyrin with the 43,000-dalton fragment preparations were measured in solution.

We looked specifically for trivial causes of reduced ankyrin binding capacity but were unable to detect an artifact that could explain our data. SDS-PAGE failed to show significant deficiencies or abnormalities of the individual peptides, and no significant proteolysis was apparent. There was <5% persistent hemoglobin, spectrin, or ankyrin on the ankyrin-stripped, inside-out vesicles, and this could not account for a reduction in binding capacity (due to persistent occupation of binding sites or due to an increase in the proportion of right-side-out vesicles). Also, ^{125}I-spectrin binding was measured to inside-out vesicles prepared simultaneously from the same erythrocytes from which ankyrin-stripped, inside-out vesicles were prepared, and these experiments demonstrated that patient and control vesicles contained identical spectrin binding capacities (a function of total ankyrin content). Besides performing the spectrin and ankyrin binding assays on erythrocytes from these three patients, a relative with classical elliptocytosis, normal relatives, and unrelated normals, we investigated erythrocytes from patients with spherocytosis and other patients who had undergone splenectomies. Reduced ^{125}I-ankyrin binding was found only in erythrocytes from these three patients, and it does not appear to be an artifact of cell size, reticulocyte count, or due to absence of the spleen.

Our conclusion that the number of high affinity ankyrin binding sites is reduced is based upon interpretation of curvilinear Scatchard plots. The patient and control membranes bound ^{125}I-ankyrin differently (Fig. 4) with individual values distinctly

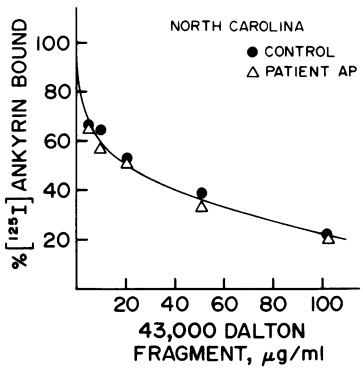

FIGURE 5 Inhibition of binding of [^{125}I]ankyrin to ankyrin-stripped, inside-out vesicles by the 43,000-dalton fragments of band 3. [^{125}I]Ankyrin and the ankyrin-stripped, inside-out vesicles were prepared from normal individuals. The 43,000-dalton fragments of band 3 were prepared from patient (△, A.P.) and control (●) vesicles by α-chymotrypsin digestion as described (32), and identical amounts of protein were obtained. [^{125}I]Ankyrin (0.4 μg, 72,500 cpm/μg) was incubated with the vesicles (9.4 μg membrane protein) and various concentrations of the 43,000-dalton fragment in 0.2 ml vol of buffer for 3 h at 0°C. Specifically membrane-bound radioactivity was determined.

different at all points in the high affinity area ($P < 0.05$ and frequently $P < 0.01$). Nevertheless, interpretations of curvilinear Scatchard plots are vulnerable to error, and it is often difficult to determine which of the parameters (B_1, K_{D1}, B_2, or K_{D2}) is responsible for apparent differences between plots (39). A change in any or all parameters would result in a different line, and it is unlikely that the calculated differences will be of statistical significance since there is potential for error at several stages in the experiments. The abscissa (B = ^{125}I-ankyrin specifically bound) includes errors in determining the total bound and non-specifically bound. Determination of unbound ^{125}I-ankyrin (F) also contains potential error. Therefore, plotting B/F vs. B results in individual points that necessarily compound several errors, and since the binding capacities (B_1 and B_2) are mutually dependent and extrapolated values, the confidence limits are invariably wide. We attempted to reduce potential errors by performing the measurements at many different points over a wide range of ^{125}I-ankyrin concentrations, using vesicles from several different patients and controls, performing the assays simultaneously, and by repeating the binding assays with fresh preparations of vesicles and ^{125}I-ankyrin on several occasions. Although computer estimates of B_1 were invariably lower for the patients than for controls, the P values within experiments were sometimes only suggestive of statistical significance ($P < 0.006$, $P < 0.10$, and $P < 0.12$). Indeed, when few points existed in the low affinity region of the curve (experiments 9A and 9B, Table IV), the confidence limits were so wide that P values were meaningless even though the calculated B_1 values were close to the values determined in other experiments (experiments 8 and 10). Nevertheless, the computer calculated values for B_1 were consistently reduced for the patients, whereas values calculated for K_{D1}, B_2, and K_{D2} were not consistently changed. Therefore, we conclude that a reduction in the number of high affinity binding sites (B_1) is the most likely explanation.

The detailed molecular explanation for a reduced number of high affinity ankyrin binding sites is not understood. Although it is clear that all ankyrin molecules bind to the cytoplasmic portion of band 3, there is a severalfold excess of potential binding sites (32, 40, 41). The reasons for this are unclear, and it is also unclear whether all ankyrin binding sites are identical. Perhaps more than one population of band 3 exists with inherent differences in the 43,000-dalton region permitting only certain populations to bind ankyrin, or perhaps all band 3 molecules have identical 43,000-dalton regions with other factors, such as favorable organization on the membrane (possibly due to the lipid phase or glycophorin), responsible for binding of ankyrin. Certain evidence suggests that the first model is less likely. The K_D of ankyrin binding to membrane and the K_I of ankyrin binding to the 43,000-dalton fragment in solution are strikingly different (32, 40). The unbound fraction of band 3 (extracted with Triton X-100) and total band 3 bind ^{125}I-ankyrin similarly when compared in solution (29) and when compared in reconstituted liposomes (40). Also, two-dimensional peptide maps of ankyrin-bound band 3 and unbound band 3 appear virtually identical (unpublished data). Some evidence suggests that band 3 exists on the membrane as a tetramer (41, 42), and it has been suggested that ankyrin may bind only to a single member of each band 3 tetramer (40). If the binding sites of our patients' cells are unfavorably clustered on the membrane, the sites available for ankyrin binding would appear diminished due to steric effects while the number of potential sites would not be reduced when cleaved from the membrane and measured in solution as in Fig. 5. Our data, like that of Bennett and Stenbuck (32), result in curvilinear Scatchard plots with apparent high affinity sites ($K_D = \sim 5$ nM) and "low" affinity sites ($K_D = \sim 0.14$ μM, which is still sufficient to result in a strong interaction). It is uncertain how these sites are related. Curvilinear Scatchard plots may not necessarily indicate a multiplicity of the types of binding sites, but may result from negative cooperativity affecting a single class of sites, or curvilinearity may result from conformational differences within a single population of receptor types. While the data of Hargreaves et al. (40) appear to result in linear Scatchard plots, significant differences in methods of ankyrin prepara-

tion, vesicle preparation (leaving up to 30% residual ankyrin), and assay technique may be responsible for this. The data contained in this report suggest that there is some difference between the two types of binding sites since only the high affinity sites are significantly reduced, and the difference does not appear to be in the 43,000-dalton fragment. Therefore, the apparent difference between high and low affinity binding sites may also be due to differences of band 3 conformation. The interaction between ankyrin and band 3 on the membrane is complicated and more investigation is required.

Dacie (43) felt that elliptocytosis represents a spectrum of disorders due to a series of defects. Our families may have a related disorder, but the inheritance patterns are unclear and may be due to various expressions of an abnormal gene even within each family. Although this report is the first to document a clinical defect of ankyrin binding sites in abnormally shaped erythrocytes, other abnormal variants of erythrocyte cytoskeletons are being discovered (16–21, 44, 45). Therefore, the defect that we have studied is probably present in only a small percentage of all cases of hemolytic anemias. Analysis of membrane protein interactions in erythrocytes from other hemolytic families may identify molecular defects responsible for other disorders. Study of these defects may provide important insight for further understanding the structure and function of cell membranes in general.

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